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Differentiation of Human Embryonic Stem Cells into Insulin-Producing Clusters

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ABSTRACT

Type I diabetes mellitus is caused by an autoimmune destruction of the insulin-producing β cells. The major obstacle in using transplantation for curing the disease is the limited source of insulin-producing cells. The isolation of human embryonic stem (hES) cells introduced a new prospect for obtaining a sufficient number of β cells for transplantation.

We present here a method for forming immature islet-like clusters of insulin-producing cells derived from hES cells.

The protocol consisted of several steps. Embryoid bodies were first cultured and plated in insulin-transferrin-selenium-fibronectin medium, followed by medium supplemented with N2, B27, and basic fibroblast growth factor (bFGF). Next, the glucose concentration in the medium was lowered, bFGF was withdrawn, and nicotinamide was added. Dissociating the cells and growing them in suspension resulted in the formation of clusters which exhibited higher insulin secretion and had longer durability than cells grown as monolayers.

Reverse transcription-polymerase chain reaction detected an enhanced expression of pancreatic genes in the differentiated cells. Immunofluorescence and in situ hybridization analyses revealed a high percentage of insulin-expressing cells in the clusters. In addition to insulin, most cells also coexpressed glucagon or somatostatin, indicating a similarity to immature pancreatic cells.

Further improvement of this insulin-producing cell protocol may lead to the formation of an unlimited source of cells suitable for transplantation. *Stem Cells 2004;22:265-274*

INTRODUCTION

Human embryonic stem (hES) cells are self-renewing pluripotent cells obtained from the inner cell mass of human blastocysts [1, 2]. Both hES cell lines and clones retain normal karyotypes even after long-term culture. The unique feature of ES cells is their dual ability to be indefinitely cultured in an undifferentiated state and to differentiate into cells representative of all three body lineages: ectoderm, mesoderm, and endoderm [1-4]. Differentiation of hES cells can be induced by removing the cells from their feeder layer and growing them in suspension. Growth in suspension leads to the formation of embryoid bodies (EBs), which are

a necessary step in the process of in vitro differentiation [3]. Such differentiation provides a new perspective for studying the cellular and molecular mechanisms of early development. In addition, their characteristics made hES cells good candidates for transplantation therapies. One of the potential uses may be the cure of diabetes.

Diabetes mellitus is a major health problem affecting more than 5% of the global population and is the most common metabolic disorder [5]. Diabetes mellitus takes two forms: type I (insulin-dependent), caused by an autoimmune destruction of the insulin-producing β cells and type II (non-insulin-dependent), resulting from a combination of reduced insulin sensitivity and impaired function of the insulin-secreting β cells. The present treatments for diabetes do not offer a cure and do not prevent the secondary complications associated with diabetes, such as diabetic retinopathy, nephropathy, and neuropathy [6]. Early treatment of these patients and restoration of the β-cell function through pancreas or islets transplantation can both free the patients from their dependency on insulin and prevent complications [7-9]. However, one of the major obstacles in human transplantation is the limited supply of donor tissue [10].

It has previously been shown that hES cells can spontaneously differentiate into insulin-producing cells, can secrete insulin, and can express other β-cell markers [11, 12]. Research conducted on mouse embryonic stem cells led to insulin-producing cells [13-16], which were capable of curing diabetes in streptozotocin-treated mice [13, 14, 16, 17]. Here we show that hES cells can be induced to form islet-like clusters similar to immature pancreatic β cells and to produce insulin.

MATERIALS AND METHODS

Cell Culture

The hES cell lines used were H9.2 (passages 45-80) [18], which is a cloned line of H9 [1], H13 (passages 45-50) [1], and I6 (passages 45-50) [19].

In Vitro Differentiation Procedure

Undifferentiated hES cells were grown on mitotically inactivated mouse embryonic fibroblast in 80% knockout Dulbecco's modified Eagle's medium (DMEM), 20% knockout serum replacement, 1 mM glutamine, 1% non-essential amino acid, 0.1 mM 2-mercaptoethanol, and 4 ng/ml basic fibroblast growth factor (bFGF) (all from GIBCO Invitrogen; Paisley, UK) (stage 1). They were then dissociated by applying 1 mg/ml type IV collagenase (GIBCO Invitrogen). Thirty minutes later, the cells were scraped with a 5-ml pipette and transferred into plastic petri dishes

(Miniplast; Ein-Shemer, Israel) to allow their aggregation. The resultant EBs (stage 2) were cultured for 7 days in 80% knockout DMEM (GIBCO Invitrogen), 20% defined fetal bovine serum (Hyclone; Logan, UT), 1 mM glutamine, and 1% nonessential amino acid (both from GIBCO Invitrogen), with a change of medium every 3 days.

Seven-day-old EBs (which consisted of 10,000 cells, on average) were plated at a density of 300 EBs per well in six-well plastic culture plates (Nunc; Roskilde, Denmark) and grown for an additional week in medium I: DMEM (DMEM)/F12 1:1, insulin (10 mg/l)-transferrin (6.7 ng/l)-selenium (ITS) (5.5 mg/l), and 1 mM glutamine (all from GIBCO Invitrogen) with a supplement of 5 μg/ml fibronectin (Roche Diagnostics GmbH; Mannheim, Germany) (stage 3). After 1 week in the ITS and fibronectin (ITSF) medium, the cells were dissociated by trypsin-EDTA (Biological Industries; Beit Haemek, Israel) to single cells and plated on plastic tissue-culture plates at a concentration of 2×10^5/ml in medium II: DMEM/F12 1:1 with N2 supplement (500 μg/ml insulin, 10,000 μg/ml transferrin, 0.63 μg/ml progesterone, 1,611 μg/ml putrascine, and 0.52 μg/ml selenite), B27 media (both from GIBCO Invitrogen), according to manufacturer's instructions, 1 mM glutamine, and 10 ng/ml bFGF (GIBCO Invitrogen). Before plating, the tissue-culture plates were coated with either 0.1% gelatin or poly-L-ornithine (15 ng/ml) (Sigma Chemical Co.; St. Louis, MO). At this stage, clusters of cells were formed (stage 4), and these cells expanded throughout the week, with change of media every other day.

At the next stage (stage 5), bFGF was removed and 10 mM of nicotinamide (Sigma Chemical Co.; St. Louis, MO) were added. Since DMEM without glucose was used, the total glucose concentration in the medium was reduced from 3,151 to 901 mg/l (medium III thus contained DMEM/F12 1:1 with 901 mg/l glucose, supplemented with N2 and B27 media, 1 mM glutamine, and 10 mM nicotinamide). After 4 days in medium III, the forming clusters were dissociated by trypsin-EDTA, and they continued to grow in suspension in petri dishes with medium III (stage 6).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from differentiated hES cells using Tri-Reagent (Sigma Chemical Co.), according to the manufacturer's recommended protocol. cDNA was synthesized from 1 μg total RNA using Moloney murine leukemia virus (MMLV) reverse transcriptase RNase H minus (Promega; Madison, WI). The absence of DNA contamination in RNA samples was confirmed with PCR primers flanking an intron. Primers and the reaction conditions are described in Table 1. The PCR products were size fractionated by 2% agarose gel electrophoresis.

Table 1. The primers used for RT-PCR, the size of the PCR fragment, and the annealing temperature

Name	Sequence	Size (bp)	Enzyme	Annealing	n of cycles
Insulin	AGCCTTTGTGAACCAACACC GCTGGTAGAGGGAGCAGATG	245	Bio-Taq (Bioline. UK)	65	40
Somatostatin	GTACTTCTTGGCAGAGCTGCTG CAGAAGAAATTCTTGCAGCCAG	179	Reddymix (ABgene, UK)	55	40
PDX1	GGATGAAGTCTACCAAAGCTCACGC CCAGATCTTGATGTGTCTCTCGGTC	230	Reddymix	65	40
Ngn3	CAATCGAATGCACAACCTCA GGGAGACTGGGGAGTAGAGG	254	Reddymix	55	40
Glucagon	AGGCAGACCCACTCAGTGA AACAATGGCGACCTCTTCTG	308	Reddymix	55	40
Pax4	GTGGGCAGTATCCTGATTCAGT TGTCACTCAGACACCTTTCTGG	308	Bio-Taq	55	40
Pax6	CCGAGAGTAGCGACTCCAG CTTCCGGTCTGCCCGTTC	239	BRL (GIBCO-BRL, UK)	65	40
Isl1	GATTTCCCTATGTGTTGGTTGC CTTCCACTGGGTTAGCCTGTAA	827	Super-Therm (JMR, UK)	60	40
Nkx6.1	GTTCCTCCTCCTCCTCTTCCTC AAGATCTGCTGTCCGGAAAAAG	381	BRL	55	40
GLUT2	AGGACTTCTGTGGACCTTATGTG GTTCATGTCAAAAAGCAGGG	231	Reddymix	55	35
PC1/3	TTGGCTGAAAGAGAACGGGATACATCT ACTTCTTTGGTGATTGCTTTGGCGGTG	457	Qiagen (Hilden, Germany)	65	40
PC2	GCATCAAGCACAGACCTACACTCG GAGACACAACCACCCTTCATCCTTC	309	Reddymix	60	40
KIR6.2	CGCTGGTGGACCTCAAGTGGC CCTCGGGGCTGGTGGTCTTGCG	497	Reddymix	65	40
SUR1	GTGCACATCCACCACAGCACATGGCTTC GTGTCTTGAAGAAGATGTATCTCCTCAC	429	Qiagen	62	40
Glucokinase	AAGAAGGTGATGAGACGGATGC CATCTGGTGTTTGGTCTTCACG	230	Reddymix	60	40
IAPP	GAGAGAGCCACTGAATTACTTGCC CCTGACCTTATCGTGATCTGCCTGC	471	Reddymix	65	40
Alpha-cardiac actin	GGAGTTATGGTGGGTATGGGTC AGTGGTGACAAAGGAGTAGCCA	486	Bio-Taq	65	35
NF-68KD	GAGTGAAATGGCACGATACCTA TTTCCTCTCCTTCTTCACCTTC	473	Bio-Taq	60	35
GAPDH	AGCCACATCGCTCAGACACC GTACTCAGCGGCCAGCATCG	302	Reddymix	60	30

Insulin Secretion

The adherent clusters obtained from this procedure were rinsed twice in Krebs-Ringer bicarbonate Hepes (KRBH) buffer (120 mM NaCl, 5 mM KCL, 2.5 mM $CaCl_2$, 1.1 mM $NaHCO_3$, 0.5% bovine serum albumin, and 10 mM Hepes) and preincubated for 2 hours with KRBH buffer containing 3.3 mM glucose. The clusters were then incubated for 1 hour in KRBH buffer with either 3.3 (low level) or 16.7 mM (high level) glucose. Different agonists and antagonist of signal pathways were added, including IBMX (3 isobutil-1-methylxanthine; 100 μM), tolbutamide (10 μM), carbachol (100 μM), and nifedipine (50 μM) (all from Sigma Chemical Co.). Insulin levels were measured using a microparticle enzyme immunoassay (AXSYM System Insulin Kit code B2D010; Abbott Laboratories; Chicago, IL), which detects human insulin with no cross-reactivity to pro-insulin or C-peptide. Protein concentrations were determined using Bio-Rad protein assay system (Bio-Rad: Hercules, CA).

Immunofluorescence

Stage VI clusters were seeded on 13-mm glass cover slides in six-well culture plates. Forty-eight hours after seeding, cells were fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized
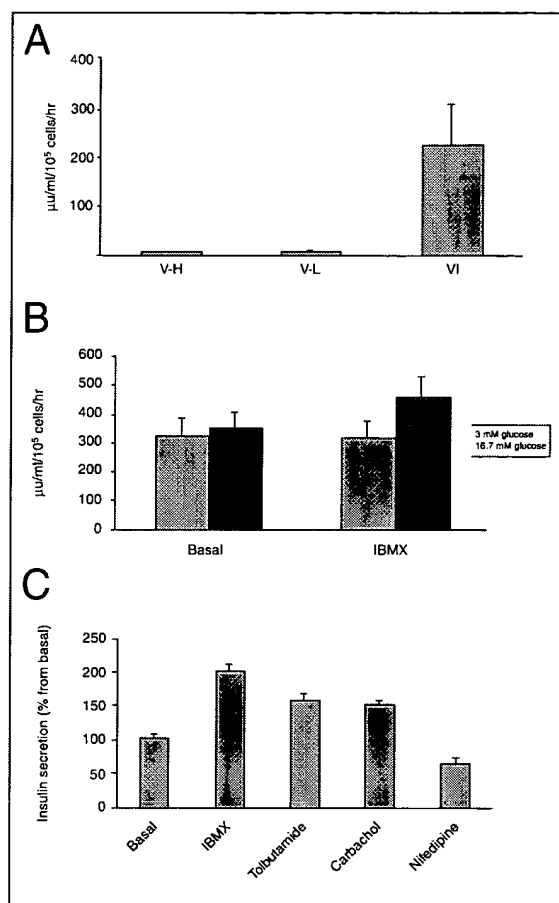
After rinsing, secondary anti-rabbit immunoglobulin G (IgG) fluorescence isothiocyanate (FITC) conjugated antibody 1:100 and anti-mouse IgG indocarbocyanine (Cy3) conjugated antibody 1:100 (both from Sigma Chemical Co.) were added to the samples, which were then incubated for an additional hour. Finally, the cells were rinsed once more and mounted with mounting media (VECTASHIELD; Vector Labs; Burlingame, CA). The slides were analyzed using a confocal microscope (Bio-Rad MRC 1024; Richmond, CA).

BrdU Labeling

To determine the percentage of proliferating cells, the BrdU Streptvidin-Biotin Labeling Kit (Zymed; San Francisco, CA) was used. The cells were incubated overnight with the BrdU labeling reagent (diluted 1:100). After the incubation, cells were rinsed 2x PBS, fixed with 75% alcohol (20 min, RT), and then stained according to the manufacturer's recommended protocol. Secondary anti-mouse IgG Cy3 conjugated antibody 1:100 (Sigma Chemical Co.) was added to the samples, which were then incubated for an additional hour. Finally, the nuclei of the cells were stained with TO-PRO-3 iodide 1:500 (Molecular Probes; Leiden, Netherlands) and mounted with mounting media (VECTASHIELD). The slides were analyzed using a confocal microscope (Bio-Rad MRC 1024).

TUNEL

TUNEL assay was performed to detect the amount of apoptotic cells within the clusters. Cells on glass coverslips were fixed for 20 min in 4% paraformaldehyde in PBS and then washed three times in PBS. Staining was performed using the In Situ Cell Death Detection Kit (Roche; Mannheim, Germany) according to the manufacturer's recommended protocol. The reaction was stopped using 2XSSC buffer, washed, and then subjected to immunofluoresence staining with mouse anti-insulin (Sigma Chemical Co.).

Antisense RNA Probe for In Situ Hybridization

Antisense RNA probe was prepared from mouse insulin I (RsaI-EcoRI) cloned into pBluescript SK[+] amp[+], which was kindly provided by Dr. S. Efrat. Following linearization, antisense RNA was transcribed using a (T7/T3) Dig-RNA labeling kit (Roche), according to company instructions. The sense RNA probe was used as a negative control.

In Situ Hybridization Analysis

Cells were seeded on poly-L-lysine-coated slides, washed with PBS at 37°C, fixed at room temperature for 30 min in a solution of 4% weight/volume (w/v) formaldehyde, 5% (v/v)

Figure 1. A) Insulin release in response to 3.3 mM glucose in different growth conditions. Left bar: insulin release from stage V-H cells (high glucose DMEM/F12 supplemented with N2, B27, and nicotinamide). Middle bar: stage V-L cells (low glucose DMEM/F12 supplemented with N2, B27, and nicotinamide). Right bar: clusters at stage VI (with the same medium as stage V-L). B) The effect of IBMX on insulin secretion. Cells were incubated with either 3 mM glucose (left bar) or 16.7 mM glucose (right bar) for 60 min with or without IBMX (100 μM). C) The effect of agonists and antagonists on insulin secretion in response to 16.7 mM glucose. The effect of IBMX, tolbutamide, carbachol, and nifedipine on the insulin secretion was determined and expressed as the percentage of the basal insulin secretion. The data of all experiments are expressed as the mean ± SE of three experiments performed in triplicates.

using 0.5% Triton X-100 in PBS/1% serum, and incubated overnight with the primary antibody mouse anti-insulin 1:100 (Sigma Chemical Co.) with either rabbit anti-C-peptide 1:100 (Linco Research Inc.; St. Charles, MI), rabbit anti-glucagon 1:100, or rabbit anti-somatostatin 1:200 (both from DAKO Corporation; Carpinteria, CA). Mouse anti-nestin 1:10 (R&D Systems Inc.; Minneapolis, MN) and mouse anti-smooth muscle actin 1:20 (DAKO Corporation) were also used with rabbit anti-C-peptide (as previously described).

acetic acid, and 0.9% (w/v) NaCl, and washed with PBS at room temperature. Prior to in situ hybridization, cells were first dehydrated by successive incubation in 70%, 90%, and 100% ethanol; then rinsed in 100% xylene; and finally rehydrated by successive incubation in 100%, 90%, and 70% ethanol. Last, the fixed cells were incubated in PBS, treated at 37°C with pepsin (GIBCO Invitrogen) for 5 min, washed with PBS, post-fixed with 1% formaldehyde for 10 min, and washed again with PBS. Prehybridization was performed for 10 min in 2XSSC, followed by 1 hour in hybridization buffer composed of 50% formamide, 0.5 mg/ml salmon sperm DNA, 4XSSC, and 1× Denhardt's reagent. Hybridization was conducted overnight (18 hours) at 42°C in maximal humidity with a 2- to 5-ng/μl digoxygenin-labeled probe. At the end of the incubation, slides were rinsed in SSC at increasing stringency conditions and re-rinsed with 0.1 Tris and 0.15 M NaCl, pH 7.5. Hybrids were detected by rhodamine conjugated anti-dig antibody (Roche).

RESULTS

Previous works using mouse [13-16] and hES [11] cells have shown their differentiation potential toward insulin-secreting cells. To promote the differentiation of hES cells into insulin-secreting cells, the procedure was performed according to a previously described protocol [15] with some modifications (Fig. 2).

The clusters formed at the end of the process were examined for their insulin-secretion potential (Fig. 1A). Decreasing the glucose concentration in the growth medium from 3,151 to 901 mg/l during stage V (as described in **Materials and Methods**) resulted in an increase in the insulin secretion in response to glucose, from 2.13 ± 1.32 to 8.64 ± 3.42 μu/ml per 10^5 cells/hour. A more dramatic increase in insulin secretion to 225.8 ± 78 μu/ml per 10^5 cells/hour was obtained by the formation of clusters at step VI (Fig. 1A). The time course of insulin secretion in response to glucose was further examined by incubating the cells for 5-60 min, which revealed that the amount of secreted insulin accumulated in the medium increased with time, reaching maximum effect after 60 min. To determine whether these cells use physiological signaling pathways to regulate insulin release, we examined the effect of several agonists and antagonists on insulin secretion. Incubating the cells with high glucose (16.6 mM) and 100 μM IBMX (3-isobutyl-1-methylxanthine; an inhibitor of cyclic-AMP phosphodiesterase) resulted in a small 40% increase of insulin secretion as compared with 3.3 mM glucose (Fig. 1B).

Some other agonists and antagonists also had an effect on insulin secretion. Tolbutamide (10 μM), an inhibitor of the K_{ATP}±-channel, and Carbachol (100 μM), an agonist of muscarinic cholinergic receptors, increased insulin secretion by 59.6% ± 5% and 50.7% ± 3%, respectively. Nifedipine
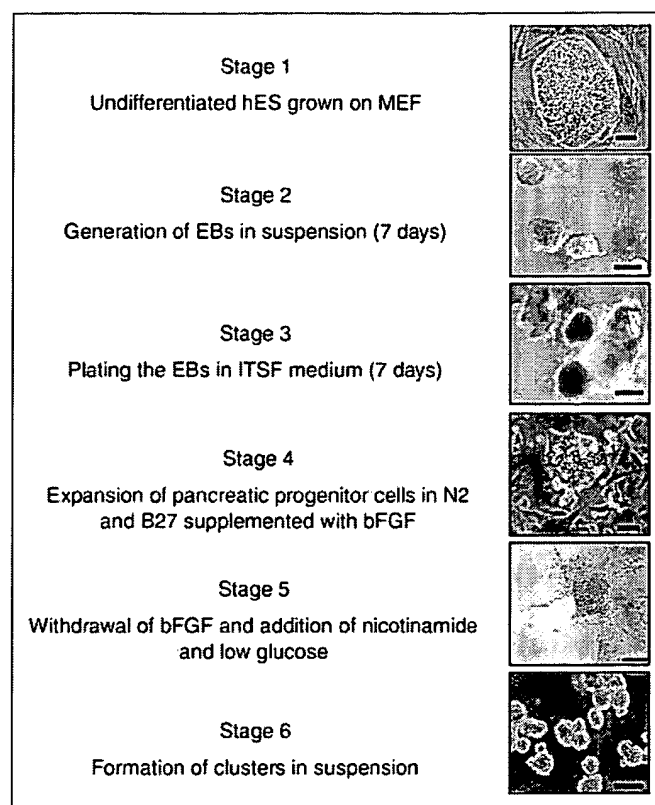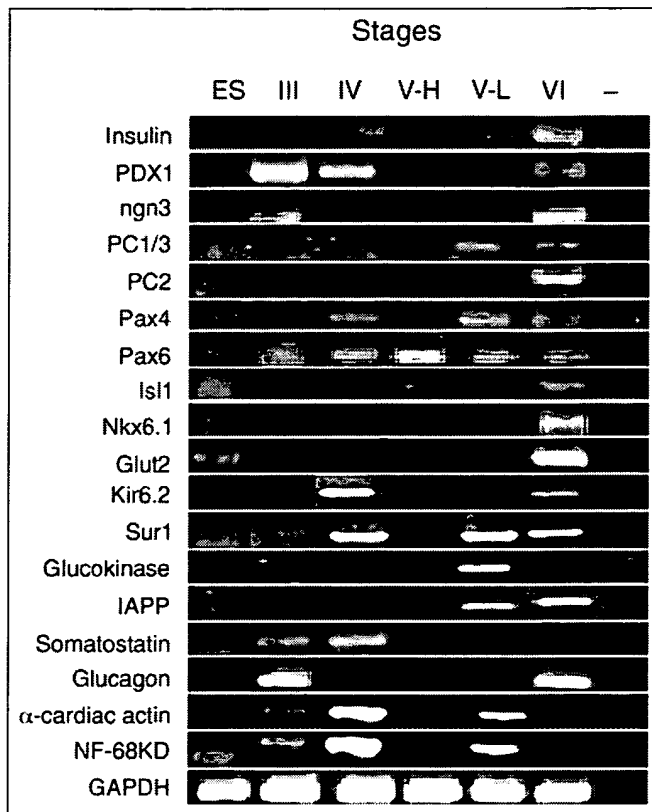

Figure 2. General outline of the differentiation protocol. The protocol consists of several stages. Stage 1: growth of undifferentiated hES cells (bar = 10 μM). Stage 2: formation of EBs (bar = 30 μM). Stage 3: plating EBs in medium I (DMEM /F12 1:1, insulin-transferrin-selenium-fibronectin and 1 mM glutamine) for 1 week (bar = 30 μM). Stage 4: dissociating the cells and plating them in medium II (DMEM/F12 1:1 with N2 and B27 media, 1 mM glutamine, and 10 ng/ml bFGF) for 1 week (bar = 5 μM). Stage 5: change to medium III (removal of bFGF, addition of 10 μM nicotinamide, and reduction of the glucose concentration from 3,151 to 901 mg/l) (bar = 10 μM). Stage 6: dissociation of the cells and growing them in suspension in petri dishes with medium III (bar = 10 μM).

(50 μM), a blocker of Ca^{2+} channels present in β cells, inhibited insulin secretion by 37.5% ± 8% (Fig. 1C). These results indicate that the pancreatic machinery is used for glucose-mediated insulin release.

RT-PCR reaction, as shown in Figure 3, demonstrated an enhanced expression of pancreatic genes in the differentiating hES cells. The transcription factor, pancreatic duodenal homeobox 1 (PDX1), appeared mainly in stage III mRNA, decreased in stage IV, and reappeared in stage VI. Glucagon and neurogenine 3 (Ngn3) were highly expressed in stage III and then in stage VI. Somatostatin expression was higher at stage IV than at stage III and then disappeared. Pax4 expression increased from stage III to IV and then decreased in stage V-H (high glucose in the medium), but when the glucose concentration was reduced, Pax4 was increased (stage V-L, low glucose in the medium and stage VI). Pax6 was noticed in all

Stages

	ES	III	IV	V-H	V-L	VI	–
Insulin							
PDX1							
ngn3							
PC1/3							
PC2							
Pax4							
Pax6							
Isl1							
Nkx6.1							
Glut2							
Kir6.2							
Sur1							
Glucokinase							
IAPP							
Somatostatin							
Glucagon							
α-cardiac actin							
NF-68KD							
GAPDH							

Figure 3. RT-PCR analysis of pancreatic gene expression in several differentiation stages. Total RNA isolated from both undifferentiated hES cells and differentiating cells was subjected to RT-PCR analysis with primers for the indicated genes. Lane 1: undifferentiated hES cells. Lane 2: stage III cells grown in ITSF. Lane 3: stage IV cells grown in DMEM/F12 with N2, B27, and bFGF. Lane 4: stage V-H cells grown in DMEM/F12 medium with N2, B27, and nicotinamide and high glucose. Lane 5: stage V-L cells grown in the same conditions as stage V-H but with low glucose concentrations. Lane 6: stage VI cells grown in suspension in the same media as stage V-L. Lane 7: represents no RTase in the PCR reaction.

results revealed a high percentage of insulin- (70%), somatostatin- (43%), or glucagon (50%)-expressing cells in the clusters. The cells coexpressed both insulin and C-peptide, indicating an undeniable production of insulin. A substantial number of the cells were costained for insulin and glucagon or somatostatin, suggesting that these cells resemble immature pancreatic endocrine cells. This phenomenon has been previously described in the development of the mouse and human pancreas [20-22]. When staining the cells for the neuron marker nestin, only 10% were stained positive. Likewise, staining for the detection of smooth muscle cells resulted in only a 5% positive staining (data not shown). Similarly, RT-PCR analysis revealed that transcripts for neuronal marker NF-68KD and cardiac actin were detected only in stages IV and V-L and were absent in stage VI cells (Fig. 3).

Cell proliferation was further tested using a BrdU staining kit (Zymed). BrdU was incorporated into proliferating cells at the S-phase. At stage VI, 20% of the cells were stained with BrdU, thus confirming that the clusters were still proliferating (Fig. 6A). Furthermore, the aggregation step increased the survivability of the cells, from 1 week at stage V to over 1 month at stage VI.

The cells were further tested with TUNEL+ combined with insulin staining. Some of the cells were stained positive for both TUNEL and insulin, suggesting apoptosis. However, in contrast to a previous work [23], 80% of the cells from stage IV that stained positive for insulin were not apoptotic (Fig. 6B). BrdU staining, TUNEL and C-peptide staining of the cells, confirmed that stage VI cells still proliferate and produce de novo insulin.
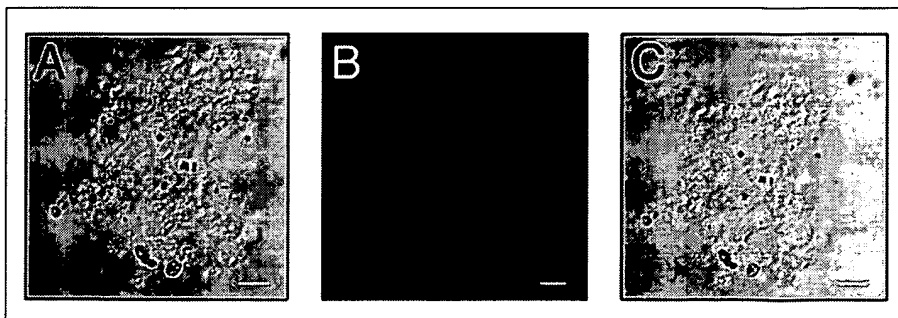
stages of differentiation. The K_{ATP}-channel genes *Kir6.2* and *SUR1* were expressed in stages IV and in stage VI, while *SUR1* was detected in stage V-L as well. Islet amyloid polypeptide (IAPP) and prohormone convertase 1/3 (PC1/3) expressions increased in stages V-L and VI. Glucokinase (GCK) was detected only in stage V-L. Insulin and other pancreatic β-cell-specific genes, such as *Nkx6.1*, *Isl1*, *Glut2*, and prohormone convertase 2 (*PC2*), were only noticed in mRNA from stage VI cells. These results indicate that differentiated cells have the ability to transcribe and process insulin.

We further examined insulin gene expression by using the in situ hybridization technique and were able to ascertain its distribution in stage VI cells (Fig. 4). Stage VI clusters were examined by immunofluorescence for the expression of insulin, C-peptide, somatostatin, and glucagon (Fig. 5). The

Figure 4. mRNA detection of insulin using in situ hybridization. Stage VI cells were hybridized with dig-labeled insulin probe, as described in Materials and Methods. Detection was performed using rhodamine-conjugated antidioxigenin antibody. A) Phase contrast image of stage VI cluster. B) Insulin mRNA in the same cells. C) Overlap of A and B showing cells stained in red as part of the whole cluster. Bar = 10 μm.
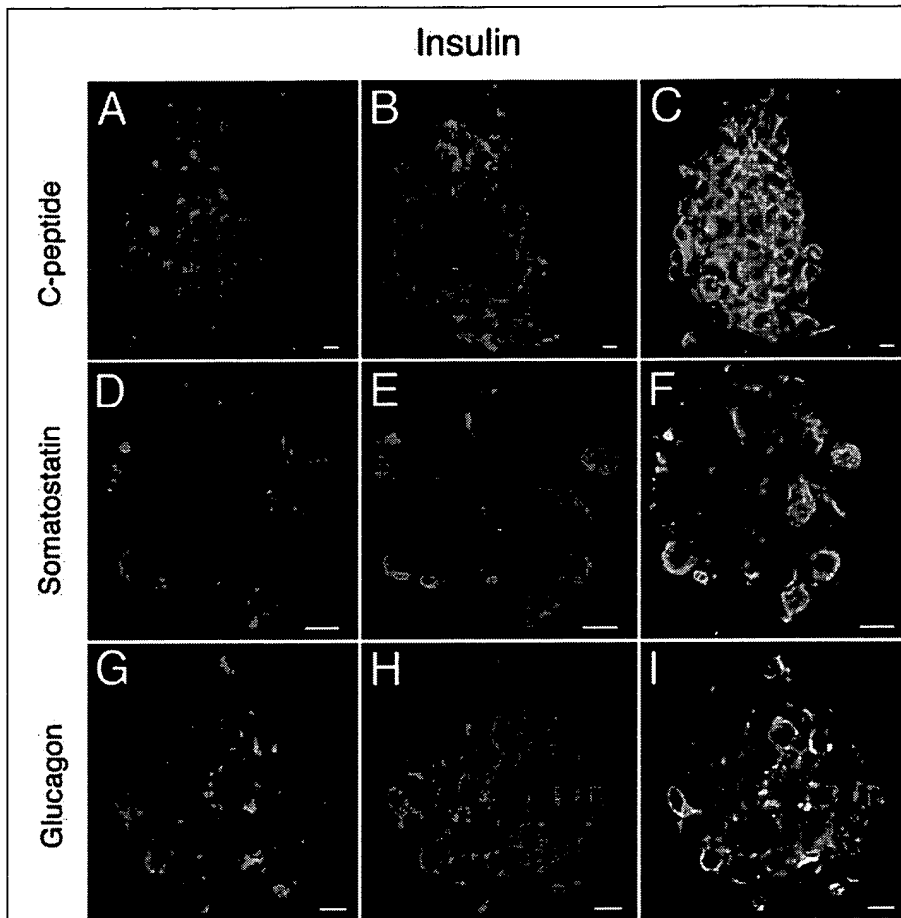
Figure 5. Confocal microscopy of immunostaining for insulin, C-peptide, somatostatin, and glucagon. Immuno-fluorescence staining of stage VI clusters. Left panel: A represents C-peptide staining, D represents somatostatin staining, and G represents glucagon staining (all in green). Middle panel: B, E, and H represent insulin staining (red). Right panel: C, F, and I represent costaining of insulin and the second marker (C-peptide, somatostatin, and glucagon, respectively). Overlapping costaining was seen as orange. Slides were analyzed using a confocal microscope. Bar = 10 μm.

Lumelsky et al. [15] modified the culture conditions used for the generation of nestin-positive cells and were able to differentiate mouse ES cells into islet-like clusters. The cells created by this method were able to produce and secrete insulin but only in small amounts per cell compared with the normal islets of Langerhans [15]. This protocol consists of several steps. One of the first stages contains ITSF that enriches the nestin-positive cells.

DISCUSSION

Previous works using hES cells demonstrated the expression of pancreas cell markers using RT-PCR [11, 12], immunohistochemistry, and insulin secretion in the differentiating cells [11]. Mouse ES cells were used in several other works, using two different strategies. The first uses the genetic cell-trapping approach [16], which was previously described for selection of cardiomyocytes [24]. Using this approach, *Soria et al.* were able to create an insulin-secreting cell clone by introducing antibiotic resistance under the insulin promoter's control. The second strategy uses the multistep differentiation approach.

Next, the cells were transferred to medium containing N2, B27, and bFGF. Both bFGF and keratinocyte growth factor were previously found to enhance the formation of islet-like clusters from ES cells [12, 15, 25, 26]. At the end of this stage, bFGF was withdrawn from the medium and nicotinamide was added. Nicotinamide is a poly-synthetase (ADP-ribose) inhibitor known to differentiate and increase β-cell mass in cultured human fetal pancreatic cells [27]. This stage induces differentiation into insulin-secreting cells. *Rajagopal et al.* [23] claimed that the insulin secretion of the cells resulted from an uptake of insulin from the culture media, and thus challenged prior works. A recent study by *Vaca et al.* [28] supports our results, showing an increase of insulin secretion after cell differentiation.
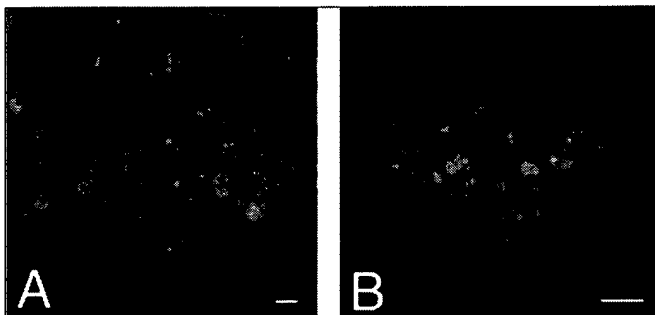

Figure 6. A) In order to determine the percent of the proliferating cells, BrdU Streptvidin-Biotin labeling was used followed by anti-mouse IgG Cy3 (red) conjugated antibody. Nuclei were stained with TO-PRO-3 iodide. Slides were analyzed using a confocal microscope. Bar = 10 μm. B) Stage 4b clusters staining for TUNEL+ and Insulin. Stage 4b cells were stained for TUNEL+ nuclei (green) with In Situ Cell Death Detection Kit followed by Immunofluoresence staining with mouse anti-insulin (red). Slides were analyzed using a confocal microscope. Bar = 10 μm

The same insulin concentration in the medium was used for stages IV to VI, yet with an increase of insulin secretion. In addition, transcript for insulin was shown both by RT-PCR and in situ hybridization, along with transcripts for proinsulin and processing enzymes, PC1/3 and PC2, indicating that stage VI cells acquired the ability to synthesize and process proinsulin to mature insulin. Transcripts for the two components of the K^+_{ATP} channel, SUR1 and KIR6.2, and for Glut2 and glucokinase, which participate in signal-secretion coupling in pancreatic β cells, were also detected. In addition, transcription factors Nkx6.1, Is11, Pax4, Pax6, Ngn3, and IAPP were also present in stage VI cells.

Somatostatin transcript was not detected beyond stage IV, although the protein was detected by immunohistochemistry in stage VI. We have found higher percentages of somatostatin-expressing cells in earlier stages (data not shown), and the staining seen in stage VI may be the result of remaining proteins. In contrast, though present in stage III and stage IV cells, only minor expression of PDX1 was observed in stage VI cells. PDX1 expression during stages III and VI may initiate a cascade of events leading to insulin transcription. Further improvement of the protocol may result in an increase in the PDX1 expression and a further increase in insulin expression and secretion.

In this study we show an improvement of the method described by *Lumelsky* [15], by A) reducing the glucose concentration in the last steps (*medium III*), and B) introducing the aggregation step, which contributes to the enrichment of insulin-producing cells and insulin secretion. The low glucose combined with nicotinamide has previously been suggested to increase the insulin content in β cells differentiated from mouse ES cells [16, 17]. The use of a cluster phase has been previously demonstrated by *Zhao et al.* [29] and others [30, 31] as a useful method for long-term maintenance of human islets in vitro. Using repeated cycles of aggregation and plating of cells obtained from postmortem human islets *Zhao et al.* [29] were successful in maintaining the cells for 4 months without losing their potential to express insulin.

Although descriptions of the clustering technique have been made in the past, they all dealt with culturing donor islet cells in vitro. This work introduces the aggregation step, which not only increased the percentage of the insulin-expressing cells but also improved their survivability from 1 week at stage V to 1 month or more at stage VI. Different studies published on cultured human islets have described a progressive loss of insulin secretion with time [29, 32], in correlation with a poor long-term survival rate of β cells in culture and overgrowth of fibroblastoid cells [32]. The poor long-term survival rate was explained by apoptosis and necrosis [32-36]. Using BrdU staining, TUNEL, C-peptide

staining, and in situ hybridization for insulin in the clusters, we were able to confirm that stage VI cells still proliferate and produce de novo insulin. Several studies dealing with differentiation of mouse ES cells toward insulin-producing cells have shown different results of insulin secretion. *Lumelsky et al.* [15] reported an insulin secretion of 3 ng/mg protein after incubating the cells for 5 min with 20 mM glucose. Similar results were recently published by *Moritoh et al.* [37]. *Blyszczuk* [14] transfected mouse ES cells with Pax4 and used a differentiation protocol to reach an insulin secretion of 20 ng/mg protein after incubating the cells with either 27.7 mM glucose or 5.5 mM glucose, with the addition of 10 μM tolbutamide. *Soria et al.* [16] reached high insulin secretion from differentiated mouse ES that were selected by using the insulin promoter (318 ng/mg protein).

Our study demonstrates that insulin secretion obtained after aggregating the cells was more than 30-fold higher than that obtained by the monolayer cells (225.8 ± 78.5 μu/ml per 10^5 cells per hour after incubation with 3.3 mM glucose). The cells showed a response to the different signal pathway agonists and antagonists. IBMX, tolbutamide, and carbachol increased the secretion of insulin, whereas nifedipine inhibited it. These results indicate that pancreatic machinery is used for glucose-mediated insulin release. The responsiveness to the glucose and to the antagonists was lower than expected, possibly due to the immaturity of the cells. These results correspond with those obtained by *Weinhaus et al.* [38], describing poor responsiveness to glucose in fetal pancreatic β cells. It seems that a cluster culture environment encourages cell-to-cell communication, as clustering cells share a greater surface contact area than those in monolayers. In addition to the enhanced insulin secretion after aggregating the cells, the percentage of insulin immunostaining was higher than in cells grown as monolayers (stage VI cells as compared to stage V cells).

A substantial number of the cluster cells formed in this work were costained for insulin and glucagon or somatostatin, suggesting that these cells resemble immature pancreatic embryonic cells. This phenomenon has been previously described in the development of the mouse [21] and human pancreas [20]. In the mouse embryo, cells expressing both insulin and glucagon were detected on day 9.5 [21]. *Polak et al.* [20] found that most 8-week-old human pancreatic cells express insulin together with glucagon and somatostatin. The percentage of cells expressing more than one marker decreased in the following weeks. One hypothesis to explain this phenomenon claimed that progenitors that transiently expressed two or more hormones gave rise to mature hormone-secreting cells [21]. Conversely, by tagging cells that expressed specific hormones with the Cre-LoxP system, it was established that double-positive progenitors do not give

rise to differentiated islet cells [39]. Thus, insulin- and glucagon-expressing cells appear to rise independently during ontogeny, probably from a common precursor [39, 40].

Because of the similarities between β cells and neuro-epithelial development [41], a similar transient expression of nestin was proposed to occur in human insulin-producing β-cell precursors [42, 43]. However, recent studies using nestin-positive cells concluded that nestin is not a specific marker of β-cell precursors, though it may contribute to microvasculature [44, 45]. Since other reports [46] have suggested that signals from endothelial cells are critical to the growth of pancreatic endocrine cells during embryogenesis, it is possible that nestin-positive cells contribute to the developing vasculature of the pancreatic mesenchyme. Thus, the proliferation of nestin-positive cells and β cells may be connected.

During embryogensis, cells expressing Ngn3 are the islet progenitors [47], and those Ngn^+ cells give rise to the endocrine cells of the pancreas. Other reports [22] do not rule out the possibility that cells expressing glucagon, insulin, and Ngn3 together eventually become adult β cells. Thus, the clusters produced in this work, which expressed insulin, glucagon, and Ngn3, show a resemblance to embryonic pancreatic precursor cells.

SUMMARY

In this work, hES cells were modified to form insulin-producing cells. By improving a protocol that had been previously used in mouse ES cells, an enriched population of insulin-producing cells was obtained. Even though these cells secreted a substantial amount of insulin, their immunostaining and RT-PCR expression revealed a similarity to immature pancreatic cells. Further maturation protocols of the nonmature cells and in vivo studies are needed. Developing hES cells to form mature β-cell-like structures will enable the use of these cells for future cell therapy of type I diabetes.

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DEVELOPMENT

Insulin Staining of ES Cell Progeny from Insulin Uptake

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Recent reports describe derivation of insulin-containing cells from embryonic stem (ES) cells (*1–5*) and putative adult stem cells (*6–8*). Of particular note is the report that mouse ES cells efficiently form islet-like structures in vitro (*1*). Using this protocol (*1*) on five ES cell lines, both murine and human, we reproduced the finding that 10 to 30% of cells stain with antibodies to insulin. Fifty-micrometer clusters of insulin-staining cells were produced as described (*1*) (Fig. S1).

Despite antibody staining, we did not detect insulin 1 mRNA by reverse transcription–polymerase chain reaction (RT-PCR) and insulin 2 mRNA detection was weak. Multiple primers used during all five stages of the protocol (*1*) confirmed these results. RT-PCR controls detected insulin transcripts from a single pancreatic β cell among 1 million non–β cells. Insulin gene expression was also assessed in ES cells with lacZ insertions downstream of the endogenous insulin or pdx1 promoters. Only about 1/100,000 cells was X-gal–positive despite insulin antibody staining in 10 to 30% of cells. Similarly, differentiated human ES cells expressing green fluorescent protein from an insulin promoter did not show fluorescence above background (*9*). Moreover, the insulin-positive cells did not stain with an antibody for C-peptide, a byproduct of de novo insulin synthesis. Nuclei of insulin-staining cells were small, condensed, and TUNEL⁺, suggesting apoptosis (Fig. 1, A to D). Electron microscopy of differentiated cells failed to demonstrate the granules characteristic of β cells.

Differentiated insulin-positive cells were reported to contain 1 μg of insulin per mg of total protein (*1*). This is less than 0.02% of the insulin found in the media to which these cells are exposed, raising the possibility that insulin is

concentrated from the medium. ES cells differentiated in media without exogenous insulin did not stain for insulin, and differentiated ES cells

subsequently cultured in insulin-deficient media lost insulin staining. (This release of absorbed insulin may mimic genuine secretion.) Some absorbed insulin is retained for more than 3 weeks in insulin-deficient media. Therefore, the mere persistence of insulin immunoreactivity in a transplant of ES cell progeny is insufficient evidence of β cell differentiation or function.

ES cells differentiated in the presence of fluoroscein isothiocyanate (FITC)–conjugated insulin concentrate FITC-insulin in the cells that stain with antibody to insulin (Fig. 1, E and F). When medium is supplemented with FITC-conjugated albumin, a distinct cell population con-

centrates this protein. Cells unrelated to β cells also concentrate insulin: Murine embryonic fibroblasts grown in the N2-based media used in the five-stage protocol (*1*) or in medium used to culture human embryoid bodies (*2*) produce cells with TUNEL⁺ nuclei that are insulin immunoreactive.

We cannot exclude the possibility that the paucity of β cell differentiation in these cultures is due to cell line variability or suboptimal culture conditions. We do conclude that insulin staining alone can overestimate genuine β cell differentiation when exogenous insulin is present. Furthermore, RT-PCR cannot quantify the number of cells within a population that produce insulin transcripts. Several methods should be combined for reliable analysis of insulin-expression including C-peptide staining, electron microscopy, Northern analysis, in situ hybridization, metabolic labeling, demonstration of biphasic insulin secretion, and transplantation assays for β cell function that demonstrate rescue of the diabetic phenotype for more than a month.

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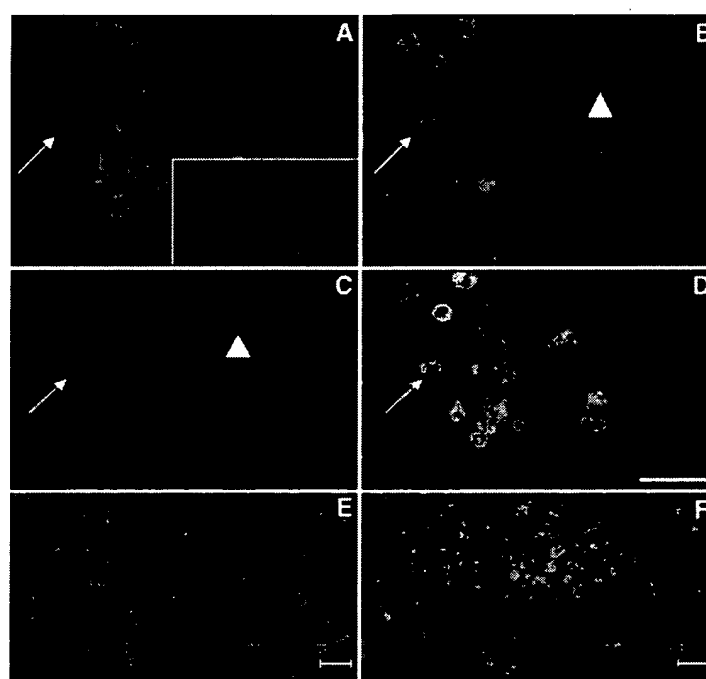*To whom correspondence should be addressed. E-mail: dmelton@mcb.harvard.edu (D.A.M.)

Fig. 1. Confocal images of insulin staining in mouse ES cell progeny. (**A**) Insulin staining (red). The inset demonstrates a typical aggregate of insulin-staining cells. (**B**) TUNEL⁺ nuclei (green). (**C**) Nuclear staining with DAPI (purple). (**D**) Composite image of (A) to (C). Cells that stain for insulin have condensed nuclei and are TUNEL⁺ (arrows). Cells with normal nuclei are TUNEL⁻ and do not stain for insulin (arrowheads). (**E** and **F**) Insulin-staining cells (red) (E) are identical to cells that have taken up FITC-conjugated insulin (green) (F). Controls showed no leakage of the FITC or rhodamine signals. Scale bars, 20 μm.